Some markers of neuronal damage in cerebrospinal fluid of multiple sclerosis patients in relapse

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Abstract
In this paper the performance of cerebrospinal fluid (CSF) protein biomarkers important for monitoring damage of brain astrocytes and neurons for MS is reviewed. We estimated neurofilament, tau and phospho-tau proteins, β-APP, Aβ, S-100B and neuron-specific enolase in CSF of MS patients during relapse. We noted elevation of neurofilament, tau and phospho-tau proteins, S-100B, neuron-specific enolase and c-terminal epitopes of β-APP; concomitantly decrease of Aβ was observed. These CSF biomarkers for MS relapse should reflect the central pathogenic processes in the brain, i.e., axonal and neuronal degeneration.

Key words: multiple sclerosis, neurofilament, tau and phospho-tau proteins, S-100B, β-APP, Aβ-amyloid protein, neuron-specific enolase.

Introduction
Multiple sclerosis (MS) is a disabling inflammatory demyelinating disorder of the central nervous system characterized by recurrent events of autoimmune-mediated demyelination and axonal loss. The disease is differential with regard to clinical course, immunological picture and radiological image. The disease usually starts with the relapsing-remitting phase, which is characterized by clinical exacerbations. The cerebrospinal fluid (CSF) is in direct contact with the extracellular space of the brain; hence biochemical changes in the brain are reflected in the CSF.

Therefore we aimed to test different neuronal biomarkers of prognostic value at the relapse stage of MS. These markers are important because they are connected with each other in metabolic processes and molecular cascades. In our study we investigated changes in protein level in CSF found as markers of neurological damage [4]. We estimated neuron-specific enolase (NSE), neurofilament light (NFL), total tau (T-tau), phospho-tau (P-tau), β-amyloid precursor protein (β-APP), amyloid β protein (Aβ/42) and glial cell marker S 100β.

As Sjögren et al. found (2001) [38], CSF NFL was increased in the group with signs of white matter changes (WMC). NFL protein is a structural component in the neuronal axons. NFL is composed of three subunits based on the molecular weight. The light subunit of NFL is located mainly in large myelinated axons. Increased CSF NFL probably reflects axo-
nal degeneration. We used antibody reacting with phosphorylated NFL [16].

Tau is a microtubule-binding protein that contributes mainly to the stability of microtubules. The binding of tau to microtubules is reduced by increases in the phosphorylation state of tau. Hyperphosphorylation of tau disrupts microtubules and leads to degeneration of neurons. In CSF tau protein is one of the biological markers establishing the degree of axonal damage in the central nervous system (CNS) [37].

The molecular cascade that follows brain damage also includes the accumulation of β-amyloid precursor protein (β-APP) and β-amyloid protein (βA/42) [1,2,18,30,39,44,45]. β-APP and βA have been shown to be multifunctional proteins which are induced as acute phase proteins by several cell types in the brain in response to injury [11]. We used antibody recognised appropriate epitopes on β-APP and βA protein.

S-100β is the calcium-binding protein localized in astroglial cells and used as a parameter of astrocyte activation and/or death in several situations of brain injury [6,29,35]. S-100β in astrocytes is found predominantly in the cytoplasm and nucleus, where it regulates cell proliferation and cytoskeleton [8,20,34]. Its physiological function is not entirely understood, but its levels are increased in the presence of central nervous system disease [25] and lesions [15].

Neuron-specific enolase (NSE) is a glycolytic enzyme that is localized primarily in the neuronal cytoplasm. In adults, CSF concentrations of NSE have served as markers of neuronal damage in patients with a variety of neurological conditions [23].

The aim of the study was to estimate CSF concentrations of markers characteristic for brain damage during relapse in MS patients.

Table I. Demographic and clinical characteristic of multiple sclerosis patients (n = 37)

| Age (years) | 35.4 ± 5.1 |
| Disease duration (years) | 6.3 ± 5.2 |
| Number of relapses | 3.4 ± 1.3 |
| EDSS before treatment (range) | 3.8 (1.0-5.5) |
| EDSS 30 days after treatment (range) | 2.2 (1.0-4.0) |
| Gender female/male ratio | 20/17 |

EDSS – Expanded Disability Status Scale (Kurtzke, 1983)

Material and methods

Sample studied

Participants in the study were patients admitted to the Neurology Department of the Medical University of Lublin.

Thirty-seven patients (20 female and 17 male) of mean age 35.2 ± 5.3 with relapsing-remitting MS (RRMS) (according to McDonald et al. 2001 [26] criteria) were consecutively studied during relapse. Relapse was defined as worsening on the Expanded Disability Status Scale (EDSS) by 1.0 point, new clinical symptoms of subjective character or objectively existing, lasting at least 24 hours, in the absence of infection or fever, after a period not shorter than 30 days of neurological status stability. Clinical disease severity was scored by Kurtzke’s Expanded Disability Status Scale (EDSS) [22]. The mean duration of clinical symptoms was 6.3 years (± 5.1). The mean number of relapses during the course of the disease in patients was 3.4 (± 1.3) (Table I).

Patients with kidney, liver, endocrine, immunological, inflammatory or infectious disorders were excluded by history, physical examination and laboratory evaluations. None of the patients had received any anti-inflammatory, immunosuppressive, immunomodulatory, steroid or hormonal treatment for at least 3 months prior to this study point.

Cerebrospinal fluid (CSF) samples were collected from patients with active MS relapse before the initiation of corticosteroid therapy.

The study was approved by the scientific ethics committee of the Medical University of Lublin, Poland.

The control group consisted of 10 age-adjusted healthy volunteers, whose CSF had been collected.

Biochemical investigation

CSF samples were concentrated by vacuum centrifugation (J W Electronic, Poland). Protein concentrations of samples were estimated by ELISA methods.

Reagents and antibodies

Monoclonal antibodies were used that recognized phosphorylated and non-phosphorylated regions of the tau molecule (Table II). Tau 14, Tau 1, and Tau 46 bind to non-phosphorylated sequences of tau. Binding of Tau 1, but not Tau 14 and Tau 46, is
Markers of neuronal damage in CSF of MS patients

Anti-bodies were purchased as follows: Tau 14 (Zymed Laboratories, CA, USA), Tau 1 (Boehringer Mannheim, Germany), Tau 46 (Santa Crus Biotechnology, Inc.). The following antibodies which recognize specific phosphorylated amino acid residues were used: AT8 (Innogenetics Laboratories, Belgium), and 12E8 (Athena Neurosciences, Inc.).

ECL Western Blotting Detection Reagent Kit (Amersham, UK) or Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, USA) was used to visualize primary antibody complexes with \( \beta \)-APP.

CSF NSE and S-100\( \beta \) concentrations were quantified by enzyme-linked immunosorbent assays (ELISA SynX Pharma Inc, Ontario, Canada) according to the manufacturer’s instructions. Samples were analysed in duplicate and compared with known concentrations of NSE and S-100\( \beta \). The lower limits of detection of the ELISA are 1.00 ng/mL for NSE and 0.01 ng/mL for S-100\( \beta \).

**Table II.** List of monoclonal antibodies used in the present studies. The location of epitopes refers to the longest tau isoform containing 441 amino acid residues [Goedert et al. 1995] [12]. Phosphate dependence is defined as a requirement for a P-Ser or P-Thr residue.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Location of epitope</th>
<th>Phosphate dependence</th>
<th>Dilution (blotting)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tau 14</td>
<td>141-178</td>
<td>None(^a)</td>
<td>1 : 1000</td>
<td>Kosik et al., 1988 [21]</td>
</tr>
<tr>
<td>Tau 1</td>
<td>199-202</td>
<td>Ser199/Ser202</td>
<td>1 : 10</td>
<td>Kosik et al., 1988 [21]</td>
</tr>
<tr>
<td>Tau 46</td>
<td>428-441</td>
<td>None(^b)</td>
<td>1 : 2000</td>
<td>Carmel et al., 1996 [5]</td>
</tr>
<tr>
<td>AT8</td>
<td>202-205</td>
<td>Ser202/Thr205</td>
<td>1 : 200</td>
<td>Goedert et al., 1995 [12]</td>
</tr>
<tr>
<td>12E8</td>
<td>262/356</td>
<td>Ser262/Ser356</td>
<td>1 : 200</td>
<td>Seubert et al., 1995 [36]</td>
</tr>
</tbody>
</table>

\(^a\)Binding is improved by the presence of microtubule binding domain [Camel et al., 1996] [5].

\(^b\)Binding is blocked by phosphorylation of Ser 199/202

**Table III.** List of monoclonal antibodies used in the present studies. For \( \beta \)-APP the following antibodies were raised against synthetic peptides corresponding to the amino acid residues of \( \beta \)-APP (697aa) isoform.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Location of epitope</th>
<th>Dilution (blotting)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb 6E10</td>
<td>597-613</td>
<td>1 : 1000</td>
<td>Kim et al., 1990 [19]</td>
</tr>
<tr>
<td>pAb RAS 57</td>
<td>C-terminal 672-695</td>
<td>1 : 1000</td>
<td>Potempska et al., 1991 [31]</td>
</tr>
<tr>
<td>( A\beta 40 )</td>
<td>597-620</td>
<td>1 : 500</td>
<td>Mehta et al., 2000 [27]</td>
</tr>
<tr>
<td>( A\beta 42 )</td>
<td>597-622</td>
<td>1 : 500</td>
<td>Mehta et al., 2000 [27]</td>
</tr>
</tbody>
</table>

The primary antibodies used against \( \beta \)-APP domain (Table III) were as follows: pAb R13 (a gift from Dr. H.M. Wisniewski, Institute for Basic Research in Developmental Disabilities (IBRDD), Staten Island, NY, USA), mAb 6E10 (Sigma), mAb-4G8 (Sigma), mAb \( \beta \)A40 (Sigma), mAb \( \beta \)A42 (Sigma), pAb RAS 57 (a gift from Dr. H.M. Wisniewski, Institute for Basic Research in Developmental Disabilities (IBRDD), Staten Island, NY, USA). ECL Western Blotting Detection Reagent Kit (Amersham, UK) or Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, USA) was used to visualize primary antibody complexes with \( \beta \)-APP.

The results were expressed as the mean ± SD (\( n = 37 \)). The statistical significance of the differences was determined by analysis of variance (ANOVA) followed by Dunnett’s test.
Results

Total tau protein levels in the patient group were 470 ± 30 pg/ml, 392 ± 72 pg/ml, 482 ± 50 pg/ml, and for the control group were 160 ± 42 pg/ml, 120 ± 34 pg/ml, 50 ± 21 pg/ml, respectively for mAbs tau 46, tau 1, tau 14. For the phosphorylated domain of tau we noted an increase of immunoreactivity of 305% up to 851% compared to control values estimated for mAb AT8 and mAb 12E8 respectively. The level of NFL was increased by 254.6% of control. Concomitantly the concentration of S-100β significantly increased by up to 8350% compared to the control value, and NSE was elevated by 4648.3% of control.

We noted significant changes in the level of β-APP immunoreactivities. The effects on the immunoreactivity, detected by the antibodies recognizing different epitopes on uncleaved β-APP molecules, were of different magnitude: the C-terminal immunoreactivity was decreased by 42%, N-terminal immunoreactivity by 18%.

The concentration of Aβ-42 was lower by 58.5% (estimated by Aβ 42 mAb), up to 71.1% (detected by 6 E10 mAb) compared with the control value. Similar results were noted for the estimated concentration of Aβ-40 epitope. We observed 48.2% and 51.8% decrease for mAbs Aβ-40 and 4G8 respectively. All results are presented in Table IV.

Discussion

In the last years there has been increased interest in the search for a potential marker for MS activity and axonal damage in this disease. The present data are compatible with the hypothesis that brain failure is compared with changes of specific biochemical markers of brain tissue activity.

Various markers have been used to establish MS activity and demonstrate axonal and neuronal damage during the disease [9,28] and also in experimental autoimmune encephalomyelitis [40]. The correlation between increased level of NSE, S-100β, NFL, tau, and phospho-tau, and decreased β-APP and βA/A4 suggested that these markers could be useful as markers in MS patients. In our study we observed that CSF total tau protein levels and phosphorylated epitopes of tau were significantly higher in MS patients compared to the controls. In MS neuronal da-

**Table IV.** List of protein biomarkers and their monoclonal antibodies used in the present studies

<table>
<thead>
<tr>
<th>Protein Biomarkers</th>
<th>Antibodies</th>
<th>MS (units)</th>
<th>Control (units)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tau</td>
<td>Tau 14</td>
<td>482 ± 50 pg/ml***</td>
<td>50 ± 21 pg/ml</td>
<td>↑ 964%</td>
</tr>
<tr>
<td></td>
<td>Tau 1</td>
<td>392 ± 72 pg/ml***</td>
<td>120 ± 34 pg/ml</td>
<td>↑ 326.6%</td>
</tr>
<tr>
<td></td>
<td>Tau 46</td>
<td>470 ± 30 pg/ml***</td>
<td>160 ± 42 pg/ml</td>
<td>↑ 293.7%</td>
</tr>
<tr>
<td>P-Tau</td>
<td>AT8</td>
<td>894 ± 45 pg/ml***</td>
<td>105 ± 33 pg/ml</td>
<td>↑ 851.4%</td>
</tr>
<tr>
<td></td>
<td>12E8</td>
<td>520 ± 70 pg/ml***</td>
<td>170 ± 20 pg/ml</td>
<td>↑ 305.8%</td>
</tr>
<tr>
<td>NFL</td>
<td>NFL</td>
<td>382 ± 160 pg/ml**</td>
<td>150 ± 70 pg/ml</td>
<td>↑ 254.6%</td>
</tr>
<tr>
<td>β-APP</td>
<td>R13</td>
<td>590 ± 40 pg/ml**</td>
<td>310 ± 32 pg/ml</td>
<td>↑ 190.3%</td>
</tr>
<tr>
<td></td>
<td>RAS57</td>
<td>690 ± 45 pg/ml***</td>
<td>350 ± 45 pg/ml</td>
<td>↑ 197.1%</td>
</tr>
<tr>
<td>Aβ-40</td>
<td>4G8</td>
<td>420 ± 80 pg/ml***</td>
<td>810 ± 40 pg/ml</td>
<td>↓ 51.8%</td>
</tr>
<tr>
<td></td>
<td>Aβ40</td>
<td>432 ± 72 pg/ml***</td>
<td>896 ± 68 pg/ml</td>
<td>↓ 48.2%</td>
</tr>
<tr>
<td>Aβ-42</td>
<td>6E10</td>
<td>320 ± 45 pg/ml***</td>
<td>450 ± 75 pg/ml</td>
<td>↓ 71.1%</td>
</tr>
<tr>
<td></td>
<td>Aβ42</td>
<td>230 ± 51 pg/ml***</td>
<td>393 ± 68 pg/ml</td>
<td>↓ 58.5%</td>
</tr>
<tr>
<td>S100β</td>
<td>S100β</td>
<td>1.67 ± 0.2 ng/ml***</td>
<td>0.02 ± 0.01 ng/ml</td>
<td>↑ 350%</td>
</tr>
<tr>
<td>NSE</td>
<td>NSE</td>
<td>152 ± 12.08 ng/ml***</td>
<td>3.27 ± 1.15 ng/ml</td>
<td>↑ 4648.3%</td>
</tr>
</tbody>
</table>

*p < 0.05
**p < 0.001
***p < 0.0001
mage takes place and tau is released into the extracellular zone, leading to increased tau levels in CSF. This is in agreement with the data of K. Blennow (2004) [3], who described increased immunoreactivity tau protein in early Alzheimer disease. The results of our study are in agreement with Rostasy et al. (2005) [33], Terzi et al. (2007) [42], and Tumani et al. (2009) [43], regarding total tau protein levels in patients with MS, and are in opposition to results published by Guimaraes et al. (2006) [14] and Jimenez-Jimenez (2002) [17], showing absence of immunoreactivity tau protein in CSF patients with MS. In our study we used antibodies for phosphorylated and unphosphorylated tau protein epitopes different than other authors. We noticed significantly increased immunoreactivity of phosphorylation epitopes compared to the controls, unlike the authors of the above-quoted articles. We think that the investigated epitopes are extremely susceptible to phosphorylation in MS and that they are early markers of axonal damage in the early stage of MS. We concluded that tau protein is a prognostic marker in the relapse stage of MS and probably reflects axonal damage.

We have also found, similar to other authors [32,38], a significant increase of the NSE and S-100β protein, which was correlated with a high level of unphosphorylated and phosphorylated tau protein.

β-amyloid, which is generated by proteolytic cleavage of the precursor β-APP, is the main protein component of plaques in the brain. β-APP is a multifunctional protein which is induced as an acute phase protein by several cell types in the brain in response to the injury [10,18]. In our investigation we detected a moderate decrease of βA42 and β-APP compared to the control value in CSF. There were small changes in CSF βA40. As a consequence, a marked decrease in the ratio βA42/βA40 was noted. CSF βA42/βA40 ratio has an important and larger diagnostic potential than CSF βA42. Our results are in agreement with results obtained by Gehrman et al. (1995) [10], who used six frozen brains of MS cases and observed the immunochemical expression pattern of APP in actively demyelinating MS lesions, and found that APP is induced on reactive glial cells and also on T lymphocytes during demyelination.

Our study was significant as it investigated the value of CSF tau protein elevation and other markers as prognostic in MS. The results presented in this study exemplify the interpretation that the pattern of different markers estimated in CSF can together reflect ongoing disease processes in the brain and is in relation to the underlying brain pathology.

**References**